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Differential Profiling of Volatile Organic Compound Biomarker Signatures Utilizing a Logical Statistical Filter-Set and Novel Hybrid Evolutionary Classifiers

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14. ABSTRACT

Volatile organic compounds (VOCs) can be monitored to reveal the identity of a unique individual, as well their physiological status. Given the analysis requirements for differential profiling via gas chromatography/mass spectrometry, our group has developed a novel informatics platform, Metabolite Differentiation and Discovery Lab (MeDDL). MeDDL's toolset identifies candidate VOCs to be used for classification. A K-nearest neighbor classifier and genetic algorithm (GA) are used to optimize the classifier and subset of VOCs. The GA uses the area the ROC curve as the optimization measure. Very promising results have been obtained on over a dozen odor recognition problems.

15. SUBJECT TERMS

machine learning, receiver operating characteristic, K-nearest neighbor, genetic algorithm, biomarker, differential profiling, gas chromatography, mass spectrometry, volatile organic compound

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Differential profiling of volatile organic compound biomarker signatures utilizing a logical statistical filter-set and novel hybrid evolutionary classifiers

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ABSTRACT

A growing body of discoveries in molecular signatures has revealed that volatile organic compounds (VOCs), the small molecules associated with an individual's odor and breath, can be monitored to reveal the identity and presence of a unique individual, as well their overall physiological status. Given the analysis requirements for differential VOC profiling via gas chromatography/mass spectrometry, our group has developed a novel informatics platform, Metabolite Differentiation and Discovery Lab (MeDDL). In its current version, MeDDL is a comprehensive tool for time-series spectral registration and alignment, visualization, comparative analysis, and machine learning to facilitate the efficient analysis of multiple, large-scale biomarker discovery studies. The MeDDL toolset can therefore identify a large differential subset of registered peaks, where their corresponding intensities can be used as features for classification. This initial screening of peaks yields results sets that are typically too large for incorporation into a portable, electronic nose based system in addition to including VOCs that are not amenable to classification; consequently, it is also important to identify an optimal subset of these peaks to increase classification accuracy and to decrease the cost of the final system. MeDDL's learning tools include a classifier similar to a K-nearest neighbor classifier used in conjunction with a genetic algorithm (GA) that simultaneously optimizes the classifier and subset of features. The GA uses ROC curves to produce classifiers having maximal area under their ROC curve. Experimental results on over a dozen recognition problems show many examples of classifiers and feature sets that produce perfect ROC curves.

Keywords: machine learning, receiver operating characteristic, K-nearest neighbor, genetic algorithm, biomarker, differential profiling, gas chromatography, mass spectrometry, volatile organic compound

1.0 INTRODUCTION

Current liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) systems typically consist of a system of specialized instrumentation with customized support software. This software is generally proprietary, being supplied by the instrument manufacturer and designed to facilitate user interaction with the analytical hardware. Most platform manufacturers also market add-on commercial software packages for the analysis of the results of GC and LC/MS experiments, which are generally designed to provide a very specific type of data analysis (i.e. proteomic or metabolomic) and cannot be readily modified or added to by the end-user. For larger metabolomic and volatile organic compound (VOC) biomarker discovery studies, such as the GC/MS based VOC profiling efforts initiated by our laboratory and collaborators, none of the software solutions reviewed[1-6] prior to development offered the ability to compare multiple time point and exposure groups, or handle data sets in significant sample numbers. This bottleneck in data handling initiated the described development and evolution of the Metabolite Differentiation and Discovery Lab (MeDDL) tool[7], allowing us to differentiate metabolite and VOC profiles in multiple differential biomarker discovery studies and facilitated the ability to visualize collected data for a global view of an entire experiment while maintaining the ability to focus on individual compounds and spectra for subsequent identification. The latest version of MeDDL incorporates a variety of additional features, described below, which focus on expanding the GC/MS analysis capability

*claude.grigsby@wpafb.af.mil; phone (937)938-3721; fax (937)656-6898;
www.wpafb.af.mil/afrl/711HPW/ of the platform in support of VOC based biomarker research on-going in our laboratory. The goal of this work was to enhance the capability of the MeDDL tool for use in differential metabolite profiling through generation of a suite of logically driven filters and machine learning tools for feature down-selection, allowing for optimally targeted unknown compound identification and potential subsequent incorporation into sensor platforms.

2.0 BACKGROUND / APPROACH

2.1 Background

Both small molecule and VOC based metabolite profiling are an attractive approach to the study of multivariate metabolic responses to such things as pathophysiological processes by which biological and chemical agents can cause perturbations in the concentrations and flux of endogenous metabolites involved in critical cellular pathways. Thus, cells and entire organisms respond to toxic insult or other stressors by altering their intra-and/or extra-cellular environment in an attempt to maintain a homeostatic intracellular environment, some of which translate to differences in measurable volatile compounds emitted. This metabolic alteration is expressed as a "fingerprint" of biochemical perturbations that may be characteristic of the type and target of a toxic insult or disease process. Additionally, if a significant number of trace molecules can be identified and monitored, the overall pattern produced may be more consistent and predictive than any single biomarker, which would prove of great value in the development of targeted sensing platforms. To illustrate our approach to these studies, we present the below urine based VOC comparison of the two parental strains of the BXD mouse model[8], C57 and DBA. This described methodology is representative of our approach and is applicable to a wide range of VOC and small molecule based biomarker discovery applications such as human performance monitoring, odor based biometrics, medical diagnostics, and targeted materials detection.

2.2 Peak Registration, Alignment, and Filtering

The MeDDL platform is an freeware informatics package currently implemented in MATLAB v2011a (The MathWorks Inc., Natick, MA) that allows for registration of “peaks,” which are defined here as a single ion or measured mass/charge (m/z) at a given retention time, mass and chromatographic time alignment, and a suite of statistical and pattern recognition tools selected for biomarker screening studies. In brief, the MeDDL tool reads in lists of CDF (common data format) conversions of the raw LC/MS and GC/MS data files, registers peaks based on user-defined parameters in terms of mass sensitivity and accuracy thresholds as well as chromatographic reproducibility tailored to the performance of the analytical platform, and performs alignment of the generated peak lists in both time and mass. Following the spectral registration and alignment previously described[7], the data was analyzed using several of the principal analytical methodologies included in MeDDL: unsupervised clustering via principal component analysis[9]; differential down selection of peaks through combination of a set of logical filters; and utilization of machine learning based tools for significant VOC "feature" identification.

MeDDL was originally created for the analysis of LC/MS data. The ionization techniques generally employed for LC/MS are termed "soft" and impart low energy to eluting ions, resulting in fairly simple mass spectra: often comprised of just the ionized analyte, or "parent" ion. Modifications to the original implementation of MeDDL were required to aid in the analysis of the more complex mass spectra in GC/MS resulting from the "hard ionization" induced by the electron impact (EI) fragmentation process in the mass spectrometer's ion source. A reductionist approach for this analysis was required for the efficient determination of changes observed between sample groups. To address this issue, we created a supplementary time-binning filter allowing the analyst to specify both a time window and lower bound threshold of peak intensities. The comparison then proceeds as follows: an averaged, composite image of each user-defined comparative group is generated (i.e. the surface obtained from samples comprising each comparative group); the most intense peak from all groups is evaluated across all aligned images using a 0.1 minute window and 100,000 absolute (total ion count) threshold; once the comparison is completed, this "time slice" based upon the peak apex ± ½ of the specified time window is removed from further analysis and the next most intense set of peaks are compared. An additional filter applied in the differential analysis of groups in this study included a fold change filter limiting results to only those peaks which demonstrated at least 2 fold or greater change in intensity between strains. It must be noted that although the MeDDL tool contains a wide variety of implemented statistical filters for feature down-selection, we limited their use to only the 2 filters listed to allow for optimal feature selection by the classifier. Once both of these filters were applied to the grouped, global data set, a Boolean "AND" was added to the resulting filtered peak sets to identify the logical intersection, an approach similar to that used in generation of a Venn diagram. These reduced data sets were then used for further classification described below.

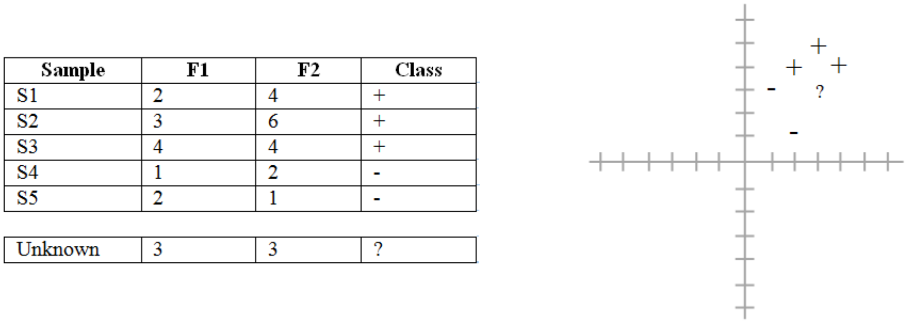
2.3 Classification

The filtered, numerical data sets, or feature vectors, produced by the preprocessing described in the previous section must be used to perform classification on unknown samples for optimal results. However, performing classification with these features still presents several problems. First, the filtered features include noisy, irrelevant features, despite the preprocessing steps taken to identify features that have both intra-class similarity and inter-class dissimilarity. Second, the set of filtered features include those that are highly correlated and therefore are redundant. These two observations suggest the classification system should produce a classifier, but should also down select the incoming feature set to a small set of cooperative features that are amenable to classification.

The following sections describe the approach used in this paper. A modified K-Nearest Neighbor (KNN) classifier is used as the basic classification algorithm. Feature selection is determined by the use of a genetic algorithm (GA) to identify a small set of features that enable the KNN to obtain good classification results. The GA is driven by the area under the KNN's receiver operator characteristic curve (ROC curve), where the ideal ROC curve has an area of 1. The following sections describe each aspect in more detail.

2.4 Modified K-Nearest Neighbor Classifier

The basic KNN is a two-class classifier that is often used in situations where the data distributions are generally unknown[10]. KNN training is performed by using all samples of the training data as labeled prototypes. Unknown samples are classified by comparing the distance of the unknown sample to the k nearest prototypes, where k is a small user-defined integer (e.g., 3). In binary classification (i.e., -two class classification), choosing an odd value for k avoids a potential tie vote. The method of computing distance with N-dimensional data is commonly done in two different ways: Euclidean distance and L1 norm, or Manhattan/Minkowski distance formula using p = 2. This work uses Euclidean distance, but the L1 norm appeared to provide similar results. The three nearest prototypes then vote on the unknown's class label. Figure 1 illustrates this process in two dimensions. In this sample, the training data contains 5 samples, which includes 3 positive samples and 2 negative samples. The three closest samples to the unknown are S1, S3, and S4, with the majority those samples being positives; consequently, the unknown would be labeled as positive.

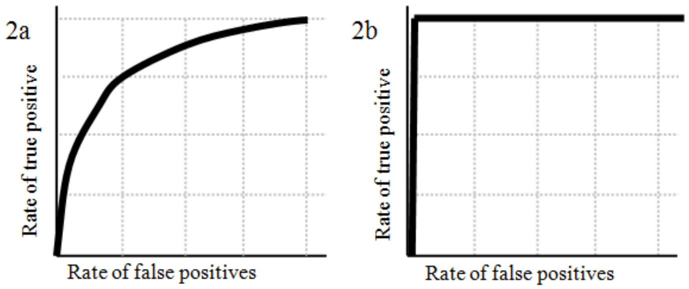


Sample	F1	F2	Class
S1	2	4	+
S2	3	6	+
S3	4	4	+
S4	1	2	-
S5	2	1	-
Unknown	3	3	?

Figure 1. Example data and plot of data.

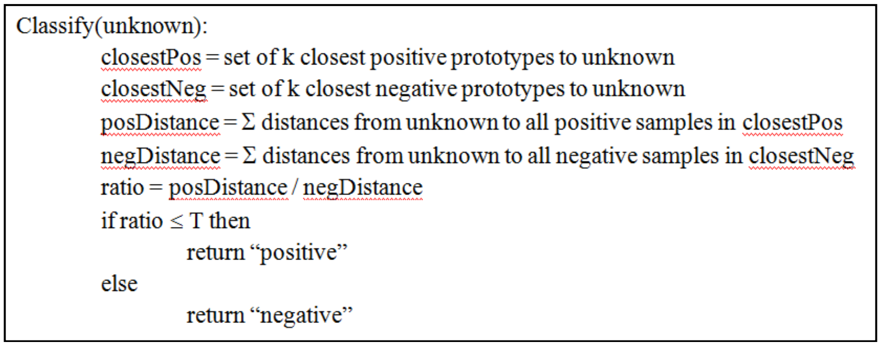
One unique objective of this work was to develop a classifier that has one or more parameters that control the classifier's behavior. For example, it may be important to correctly classify positives, with an increased tolerance for false alarms. Conversely, it may be deemed acceptable to miss a couple positives, if the increased number of false alarms is kept small. The k parameter in the KNN classifier does not provide such a parameter. k simply denotes the number of voters and does not provide a way to increase/decrease the sensitivity toward the class boundaries. Further, the number of prototypes is typically quite small in biological studies and therefore modulating the number of voters would have limited utility.

For an appropriately configurable classifier, a ROC curve visually illustrates the possible tradeoffs between the rates of true positives and false positives. Figures 2a and 2b illustrate a typical ROC curve and the perfect ROC curve. Figure 2a depicts the tradeoffs of a hypothetical classifier. The figure shows that the classifier has a parameter that can allow it to obtain a 0.75 true positive rate, while simultaneously having a false alarm rate of 0.25. Should the operational situation require 0.9 rate of recognizing true positives, the rate of false alarms would reach a predicted level of approximately 0.75. ROC curves are monotonically increasing. The perfect classifier would obtain a rate of 1.0 for positives with a false alarm rate of 0.0. This perfect ROC curve is shown in Figure 2b.



Figures 2a-b. ROC Curves. Figure 2a shows a typical ROC curve. Figure 2b shows the perfect ROC curve.

To provide for an adjustable parameter, the KNN's decision rule is modified (Figure 3). Whereas, the basic KNN's decision rule is to count the votes to the nearest k prototypes, the modified decision rule uses the distance value to influence its decision. This is approach assumes that being closer to a prototype indicates that it is more likely to be of that category. The definition for the modified KNN decision rule is as follows, where T is the configurable parameter and k is an integer > 0:



```
Classify(unknown):
        closestPos = set of k closest positive prototypes to unknown
        closestNeg = set of k closest negative prototypes to unknown
        posDistance = $\Sigma$ distances from unknown to all positive samples in closestPos
        negDistance = $\Sigma$ distances from unknown to all negative samples in closestNeg
        ratio = posDistance / negDistance
        if ratio $\leq$ T then
                return "positive"
        else
                return "negative"
```

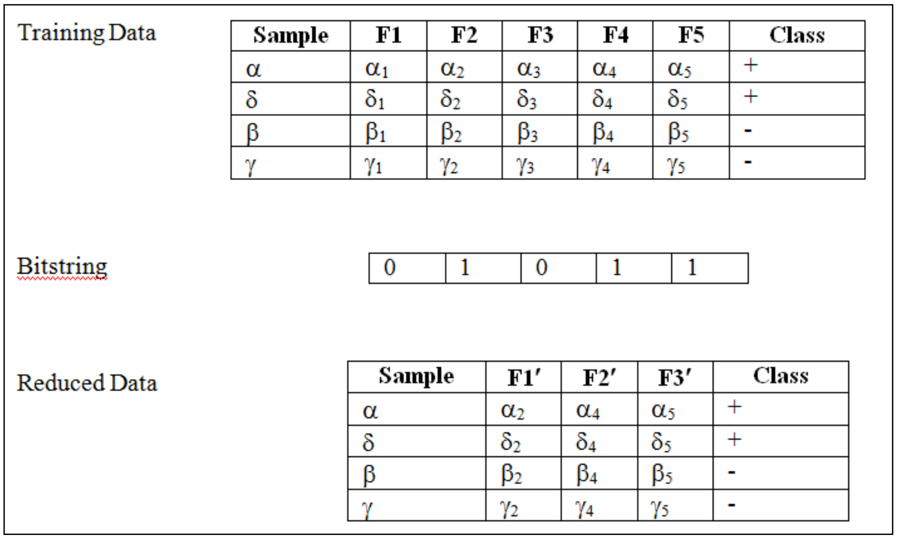
Figure 3. Modified KNN pseudocode.

The classification rule takes the ratio between the total distances to the closest positive prototypes and the closest negative prototypes. If the unknown happens to be a positive, it is expected that posDistance would be small and negDistance would be large, producing a small value for ratio. By adjusting T to a small value, the criteria for declaring "positive" becomes more stringent, in that the unknown's distance from the positives must be quite small while simultaneously its distance from the negatives must be relatively large. Conversely, setting T to a large value allows more samples to be classified as positives. In the extreme case, T = infinity, all unknown samples will be classified as positives.

2.5 Learning Algorithm for Feature Selection

After preprocessing, the set of filtered features is sent to the classification system. As mentioned in section 2.2, the potential exists for reducing this set to an even smaller number. Ideally, this reduction would produce a less costly system and produce a subset of features that are more effective than using the entire set as a whole. The ideal subset would contain features with general properties such as: mutual independence, inter-class dissimilarity, and intra-class similarity. Rather than applying more filters to achieve this, our approach is to use the modified KNN classifier to assess the quality of a feature subset; where good subsets will provide good classification and poor subsets will not be very accurate.

The process of selecting a subset from a large set uses a sequence of 0's and 1's to represent the subset. Here the bit positions containing a 1 or 0 indicate features to be included or excluded. Figure 4 shows a diagram illustrating how one bitstring is used to down-select the features and how that down-selection affects the resulting data set that is fed to the KNN learning algorithm. In this example, the bitstring happens to have three on-bits located at positions 2, 4, and 5, indicating that only features 2, 4, and 5 are used and features 1 and 3 are ignored. The down-selected data is then used to form the modified KNN.



Training Data

Sample	F1	F2	F3	F4	F5	Class
α	α_1	α_2	α_3	α_4	α_5	+
δ	δ_1	δ_2	δ_3	δ_4	δ_5	+
β	β_1	β_2	β_3	β_4	β_5	-
γ	γ_1	γ_2	γ_3	γ_4	γ_5	-

Bitstring

0	1	0	1	1

Reduced Data

Sample	F1′	F2′	F3′	Class
α	α_2	α_4	α_5	+
δ	δ_2	δ_4	δ_5	+
β	β_2	β_4	β_5	-
γ	γ_2	γ_4	γ_5	-

Figure 4. Reduction of Training Data. The topmost figure shows the entire set of data. The middle figure shows one bitstring produced by the GA. The bottommost figure shows the training data without the excluded features.

A GA is a natural learning algorithm to apply to this problem[11, 12] since it operates on a bitstring. The reader is referred to the text by Goldberg[13] for a more complete treatment of GAs. For our purposes, it suffices to say that the GA is a method for optimizing a sequence of 0's and 1's. In order to achieve this, the GA requires a method for evaluating the quality of the sequence. By assigning a numeric score to a sequence, and many other sequences, the GA navigates the search space to find sequences that are better than the ones it is currently is examining.

Leave-one-out (LOO) cross validation[10, 14] is a common method for estimating the quality of a classifier using only training data. LOO iterates over all the training samples, where each sample is temporarily removed from the training set. This smaller set is then used to train the classifier, which is then applied to the sample that was held out. Ideally, the classifier will correctly classify the sample. By repeating this process over all training samples, it is possible to assess the generality of the learning technique. If the LOO algorithm shows solid performance over a large percentage of the samples, it can be assumed that the learning technique generalizes to truly unknown samples.

On each iteration of the LOO algorithm, the bitstring in question ultimately results in a KNN that is used to classify the sample temporarily removed. Instead of classifying the sample, the ratio between posDistance and negDistance is recorded. The set of ratios can be used to create a ROC curve that predicts the final system's ROC curve, where the final system refers to the modified KNN that is obtained by using all of the training data. The area under the predicted ROC curve is used as the bitstring's evaluation score. Naturally, a score of 1 corresponds to a perfect ROC curve, which indicates that the feature set forms an effective KNN classifier.

3.0 EXPERIMENTAL DESIGN

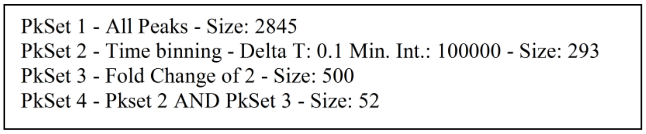
3.1 Materials / Methods

Animal use in this study was conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996, and the Animal Welfare Act of 1966, as amended. BXD mice parental strains (DBA and C57) utilized for this study were singly housed in metabolic cages which are approximately 9 cm in diameter and urine and feces were separated and isolated. Individual mouse urine samples were collected using 1 mL disposable transfer pipettes (Thermo Fisher Scientific) and placed in 2 mL Eppendorf Snap-Cap Microcentrifuge Safe-Lock tubes. The urine was then stored frozen at -80°C and thawed on ice prior to analysis. For the BXD VOC baseline set described, 170 individual samples representing the two parental strains (C57 N = 81, DBA N = 89), and six additional test samples (C57 N = 3, DBA N = 3) were processed by aliquoting 200 uL of urine into a 10 ml crimp-top headspace vial (National Scientific). The vials were immediately crimped with Red PTFE/white silicone crimp seals (Fisher). The bench-top GC/MS system utilized for sample analysis was a Thermo Fisher Trace GC Ultra gas chromatograph interfaced to a Thermo Triplus autosampler configured for automated SPME headspace sampling and in-line with a Thermo DSQII single quadrupole mass spectrometer. Collection of organic volatiles from the urine was accomplished using a 2cm CAR/DVB/PDMS solid phase micro extraction fiber (SPME), Supelco supplier, inserted by the Triplus autosampler into the head-space of the sample vials. The headspace samples were incubated at 60°C for 15 minutes, followed by extraction at 60°C for 30 minutes and automated direct injection. Volatiles gathered by the SPME fiber were analyzed through desorption of the fiber by heating to elevated temperature and separation with a Restek Stabilwax 30m, 0.25mm ID column. Helium was used as the carrier gas at a flow-rate of 1.5 ml/min. A narrow bore SPME injector liner (0.75 mm I.D.) was used (Thermo). The following conditions were utilized for sample analysis: desorption for 2 min via a PTV injector held at 230°C; oven temperature program 50°C (4 min); 5°C/min to 230°C; hold 30 minutes giving a total run time of 70 minutes. The DSQII MS transfer line was held at 230°C and the instrument was operated in positive scan mode from 41 to 400 amu. The raw data was collected in centroid mode and the resulting chromatograms and mass spectra (raw files) were then converted to CDF format and subsequently analyzed through MeDDL. Due to the fact that SPME extraction is a competitive process leading to mutual displacement from the adsorption sites between different analytes or analytes and matrix constituents, the results of this study as described report data semi-quantitatively based on relative peak heights.

3.2 Results

A total of 170 BXD parental urine samples (DBA and C57 “teaching set”) were collected and analyzed over a four month period with the six unregistered “unknown”, test samples utilized below acquired over 12 months later. Following GC/MS analysis, CDF conversion, and MeDDL registration, the samples in the “teaching set” were filtered for a 2-fold change and time binned (0.1 min window, 100K absolute threshold minimum cutoff). The filter results are shown in Table 1, with peakset 1 comprising all registered peaks, peakset 2 comprising time binning, peakset 3 comprising fold change, and peakset 4 the resultant intersection of the two applied filters.

Table 1. BXD parental strain peak registration and peakset (PkSet) filter results.



```
PkSet 1 - All Peaks - Size: 2845
PkSet 2 - Time binning - Delta T: 0.1 Min. Int.: 100000 - Size: 293
PkSet 3 - Fold Change of 2 - Size: 500
PkSet 4 - Pkset 2 AND PkSet 3 - Size: 52
```

This subset of 52 VOC features, or peaks, were first screen by PCA (Figure 5) to demonstrate group separation prior to analysis by the hybrid GA classifier. Principal component analysis is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. This technique is often difficult in usage to identify the individual subset of features responsible for group separation, but is quite useful as a screening technique as shown below.

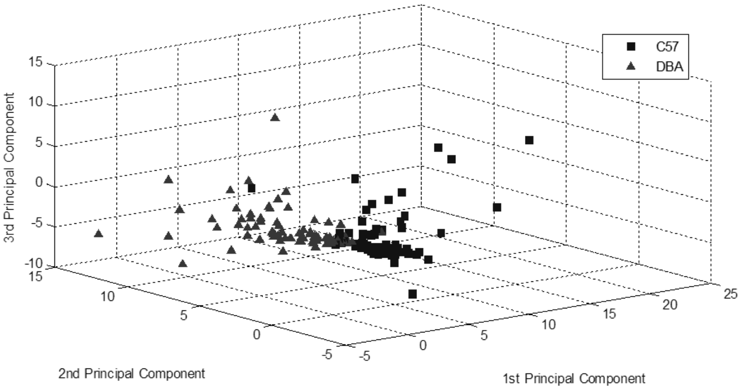
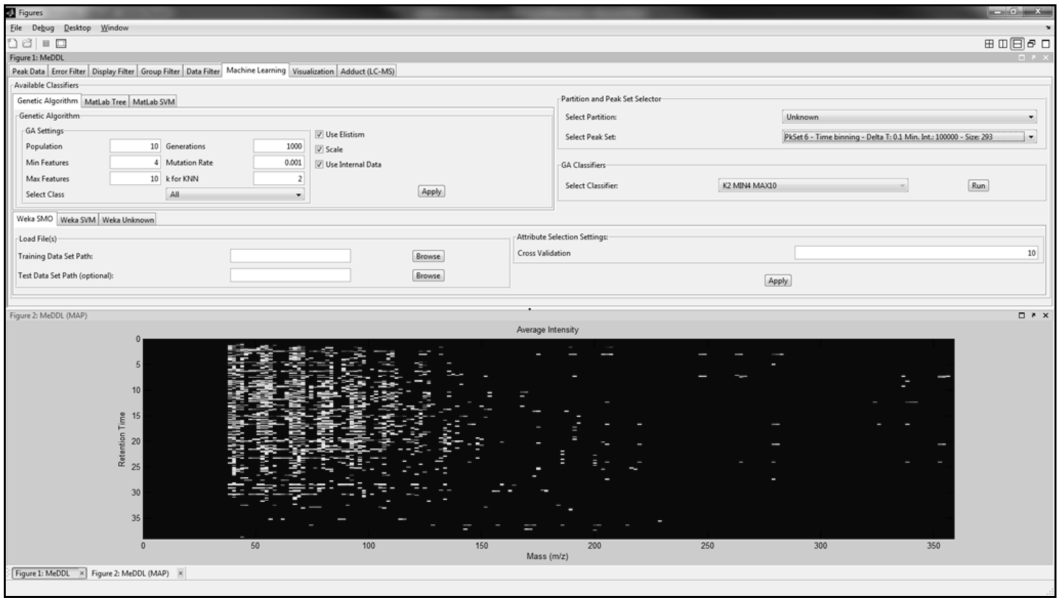


Figure 5. PCA of C57 and DBA filtered intersect (peakset 4) results.

MeDDL offers users the ability to utilize several different types of classification methods and separately store the resulting output for classification of additional, unregistered unknowns. These methods use a combination of pre-coded Matlab classifiers, Waikato Environment for Knowledge Analysis (WEKA) classifiers, and the novel, in-house developed hybrid GA classifier, implemented in Java and Matlab, described in this study (Figure 6). The internal data classification allows users to teach the classifiers from peak sets generated using the tool. The external data classification is currently designed to process both CDF format files and comma separated value files (.CSV). All classification methods support classifying intensities or ratios of intensities though application of appropriate data filters.


Figures
File Debug Desktop Window
Figure 1: MeDDL
Peak Data | Error Filter | Display Filter | Group Filter | Data Filter | Machine Learning | Visualization | Adduct (LC-MS)
Available Classifiers
Genetic Algorithm | MatLab Tree | MatLab SVM
Apply
Weka SMO | Weka SVM | Weka Unknown
Browse
Browse
Run
Apply
Figure 2: MeDDL (MAP)
Average Intensity
Retention Time
0
5
10
15
20
25
30
35
0
50
100
150
200
250
300
350
Mass (m/z)
Figure 1: MeDDL
Figure 2: MeDDL (MAP)

Genetic Algorithm

GA Settings

Population	10	Generations	1000
Min Features	4	Mutation Rate	0.001
Max Features	10	k for KNN	2
Select Class		All	

☑ Use Elistism
☑ Scale
☑ Use Internal Data

Partition and Peak Set Selector

Select Partition: Unknown

Select Peak Set: PkSet 6 - Time binning - Delta T: 0.1 Min. Int.: 100000 - Size: 293

GA Classifiers

Select Classifier: K2 MIN4 MAX10

Load File(s)

Training Data Set Path:

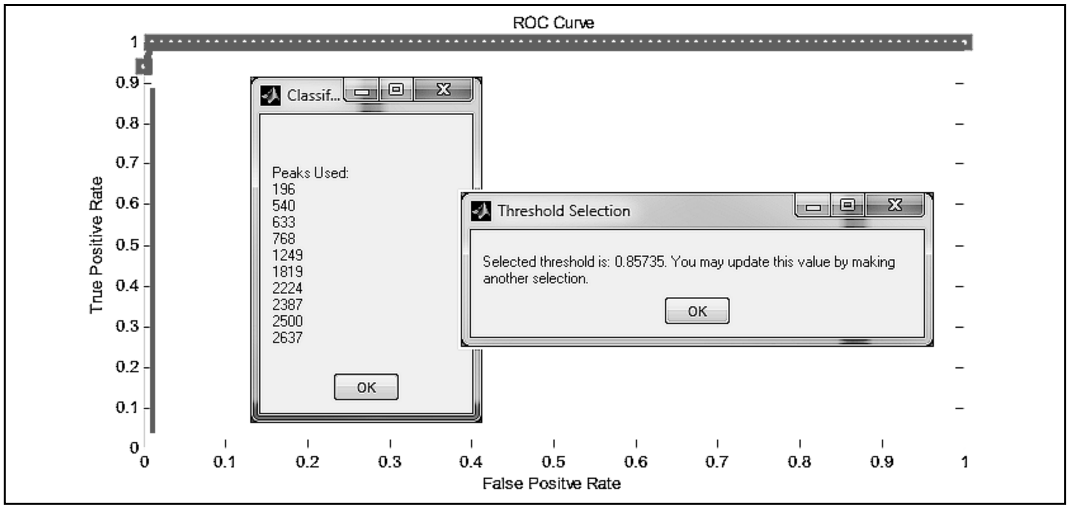
Test Data Set Path (optional):

Attribute Selection Settings:

Cross Validation 10

Figure 6. MeDDL tool machine learning implementation and GA settings.

In testing the hybrid GA for this study (Figure 7), setting k = 2, minimum features = 4, and maximum features = 10 provided both perfect classification of C57 versus DBA for both the 170 teaching samples as well as the 6 “unknown” external samples. Reverse classification (DBA versus C57) using these same settings resulted in 2 mis-classifications of the “unknowns” illustrating the need to optimize the GA settings for each classifier result.



Peaks Used:
196
540
633
768
1249
1819
2224
2387
2500
2637

Selected threshold is: 0.85735. You may update this value by making another selection.

Figure 7. Hybrid GA results. Vertical line is user adjustable slider to determine T threshold values.

Results of the hybrid GA classifier were comprised of 10 VOC “features”, which is the maximum features size allowed by the GA settings. An example of one of the selected VOCs is shown in Figure 8. In an focused biomarker study, each resultant peak would then be preliminarily identified through comparison to the National Institute of Standards and Technologies (NIST) 08 database and Wiley libraries and verified though expert, manual spectral analysis and comparison with purchased standards.

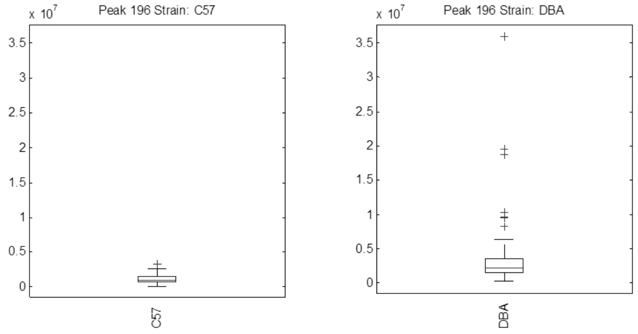


Figure 8. Boxplot of hybrid GA VOC feature output selected by classifier.

4.0 CONCLUSION

Given the unique requirements for large-scale, LC/MS and GC/MS based biomarker studies and currently available software limitations, a logically designed and successfully implemented comprehensive tool for time-series spectral registration, spectral and chromatographic alignment, visualization, and comparative analysis facilitates and allows the efficient and methodical analysis of multiple, large-scale biomarker discovery studies. The MeDDL platform has been markedly improved from the original version and greatly streamlines the analysis of multi-group comparisons through the addition of a more intuitive interface, the ability to dynamically alter group definitions and group comparative displays, and the creation of definable, group comparative graphics. Through a combination of the base MeDDL registration and alignment algorithms and the described additional functionality, MeDDL now offers the analytical chemist the potential for visualizing data in new ways, providing novel insight into the experimental results, and expediting LC/MS and GC/MS based biomarker discovery. Modifications to the current implementation of the tool are on-going, with automated iteration across available “unknowns” for optimization of the hybrid GA parameter settings planned. A compiled version of MeDDL is provided free of charge to all interested parties and is available at the following URL along with a Wiki (http://meddl.cs.wright.edu/) covering many of the features described above.

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